Table 1A. Criteria for the classification of pathogenic mt-tRNA variants. The ACMG criteria are listed on the left column and the new criteria for mt-tRNA variants are on the right.

Evidence	ACMG criteria	Criteria for mt-tRNA variants
Very strong	**PVS1** Null variant in a gene where loss of function is a known mechanism of disease.	**PVS1** Not applicable.
Strong	**PS1** Same amino acid change as a previously established pathogenic variant regardless of nucleotide change.	**PS1** Not applicable.
	PS2 *De novo* in a patient with disease and no family history.	**PS2** Present at ≥5% heteroplasmy, in ≥2 different tissues of the affected individual but 0% in asymptomatic mother. If mother's sample is unavailable, and 0% in other asymptomatic matrilineal relatives (such as proband's siblings, proband's maternal grandmother), it will be downgraded to PM9. Note: The percentage of heteroplasmy should be analyzed by dependable, clinically validated method, such as deep NGS using one-piece Long Range PCR product of the circular mtDNA template. The sensitivity of the methodology must be able to distinguish true zero from background zero.
	PS3 Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the gene or gene product.	**PS3** Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the mitochondrial function. These could be transmitochondrial cybrid assays, ETC, OCR, ATP synthesis, mtDNA copy number, COX deficient fibers, single fibers, etc, at a diagnostic levels (depending on assays and tissue types), and correlate with percentage of heteroplasmy. If not correlate with percentage of heteroplasmy, it will be downgraded to PM10.
	PS4 The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls.	**PS4** The prevalence of the variant in affected individuals is significantly increased compared to the prevalence in controls.

		PS5 Rare variants previously reported as pathogenic. Note: Not all reports are reliable, especially those old ones published before the application of deep NGS to sequence mtDNA. Please review the literature cautiously before use this criterion.
Moderate	**PM1** Located in a mutational hot spot and/or critical and well-established functional domain without benign variants	**PM1** Variants that cause anticodon swap.
	PM2 Absent from controls in Exome Sequencing Project, 1000 Genomes or ExAC.	**PM2** Absent from database, e.g., mtDB and MitoMap, and absent or low heteroplasmy （<5%） in asymptomatic mother, compared to that in the proband. If mother's sample is unavailable, it will be downgraded to PP7. Note: The percentage of heteroplasmy should be analyzed by dependable, clinically validated method, such as deep NGS, using one-piece Long Range PCR product of the circular mtDNA template.
	PM3 For recessive disorders, detected in *trans* with a pathogenic variant.	**PM3** Not applicable.
	PM4 Protein length changes due to in-frame deletion/insertions in a non-repeat region or stop-loss variants.	**PM4** Not applicable.
	PM5 Novel missense change at amino acid residue where a different missense change determined to be pathogenic has been seen before.	**PM5** Same position nucleotide change of a previously well-established pathogenic variant to a different nucleotide. Example: m.3243A>T vs m.3243A>G.
	PM6 Assumed *de novo*, but without confirmation of paternity and maternity.	**PM6** Not applicable. See PS2
		PM7 MitoTIP prediction score >16.0.

		PM8 Heteroplasmy (≥5%) among different tissues of an affected individual correlates with clinical or biochemical phenotypes. Example: The heteroplasmy of a patient's muscle is at 10%, while the heteroplasmy in blood is at 3%.
		PM9 At least two independent families, or two matrilineal family members from one family demonstrate correlation of heteroplasmy (≥5%) with clinical or biochemical phenotypes.
		PM10 Well-established *in vitro* or *in vivo* functional studies supportive of a damaging effect on the mitochondrial function. These could be transmitochondrial cybrid assays, ETC, OCR, ATP synthesis, mtDNA copy number, COX deficient fibers, ragged red fibers, etc, at a diagnostic levels (depending on assays and tissue types), **but not correlate with percentage of heteroplasmy.**
Supporting	**PP1** Co-segregation with disease in multiple affected family members in a gene definitively known to cause the disease.	**PP1** Not applicable. Upgrade to PM8 or PM9 when co-segregate not only with disease but also with >5% heteroplasmy.
	PP2 Missense variant in a gene that has a low rate of benign missense variation and where missense variants are a common mechanism of disease.	**PP2** Not applicable.
	PP3 Multiple lines of computational evidence support a deleterious effect on the gene or gene product.	**PP3** The range of MitoTIP prediction score is within [12.5-16].
	PP4 Patient's phenotype or family history is highly specific for a disease with a single genetic etiology.	**PP4** Patient's phenotype or family history is highly specific for a mitochondrial disease with a single genetic etiology.
	PP5 Reputable source recently reports variant as pathogenic but the evidence is not available to the laboratory to perform an independent evaluation.	**PP5** Not applicable.
		PP6 Heteroplasmy in an affected proband ≥5%.

	PP7 Absent from database, e.g., mtDB and MitoMap, and is heteroplasmic.

ETC: electron transport chain, OCR: oxygen consumption rate, COX: cytochrome C oxidase. mtDB: human mitochondrial genome database.

Table 1B. Criteria for the classification of benign variants.

Evidence	ACMG criteria	Criteria for mt-mRNA variants
Stand-alone	**BA1** Allele frequency is above 5% in Exome Sequencing Project, 1000 Genomes, or ExAC.	**BA1** Top-level haplogroup defining variants.
Strong	**BS1** Allele frequency is greater than expected for disorder.	**BS1** Reported in public databases (e.g., MitoMap or mtDB) or literatures as polymorphism.
	BS2 Observed in healthy adult individual for a recessive (homozygous), dominant (heterozygous), or X-linked (hemizygous) disorder with full penetrance expected at an early age.	**BS2** Found homoplasmic in more than three unrelated healthy adults.
	BS3 Well-established *in vitro* or *in vivo* functional studies shows no damaging effect on protein function or splicing.	**BS3** Not applicable.
	BS4 Lack of segregation in affected members of a family.	**BS4** Homoplasmy in both probands and at least 2 asymptomatic matrilineal family members.
Supporting	**BP1** Missense variant in a gene for which primarily truncating variants are known to cause disease.	**BP1** Not applicable.
	BP2 Observed in *trans* with a pathogenic variant for a fully penetrant dominant gene/disorder; or observed in *cis* with a pathogenic variant in any inheritance pattern.	**BP2** Not applicable.
	BP3 In-frame deletions/insertions in a repetitive region without a known function.	**BP3** Not applicable.
	BP4 Multiple lines of computational evidence suggest no impact on gene or gene product.	**BP4** The MitoTIP prediction score is $\leq$ 10.
	BP5 Variant found in a case with an alternate molecular basis for disease.	**BP5** In the presence of a known pathogenic genetic cause unless there is evidence of more than one disease and clinically

		explained.
	BP6 Reputable source recently reports variant as benign but the evidence is not available to the laboratory to perform an independent evaluation.	**BP6** Found to be homoplasmic 1-3 times in private reputable laboratory databases.
	BP7 A synonymous (silent) variant for which splicing prediction algorithms predict no impact to the splice consensus sequence nor the creation of a new splice site AND the nucleotide is not highly conserved.	**BP7** Not applicable.